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Mycorrhization of containerized *Pinus nigra* seedlings with *Suillus granulatus* under open field conditions

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Abstract

Seedling mycorrhization acts as an efficient tool for improving the quality of seedlings. In this study, the effectiveness of *Suillus granulatus,* originating from *Pinus heldreichii* forests (Montenegro), to produce containerized ectomycorrhizal seedlings of autochthonous *Pinus nigra* in open field conditions was investigated. Spore (10^6, 10^7, 10^8) and vegetative (1:16, 1:8, 1:4) inoculation on ectomycorrhizal formation and seedling growth were tested. Spore and vegetative inoculums of autochthonous *Pisolithus arhizus* were used in the same trial as additional control treatments.

The utilization of vegetative and spore inoculums of autochthonous *S. granulatus* has proven to be an effective method of obtaining containerized ectomycorrhizal *P. nigra* seedlings under open field conditions after 11 months. *S. granulatus* spore inoculations resulted in well developed ectomycorrhiza, decreasing the growth of the *P. nigra* seedlings in the first growing season. Mycelial inoculations resulted in slightly developed *S. granulatus* ectomycorrhiza, which increased the growth of the seedlings. Therefore, it would be feasible to use spore inocula of *S. granulatus,* with 10^6 spores per plant, to produce ectomycorrhizal *P. nigra* plants on a large scale.

Controlled mycorrhizal inoculation of seedlings is not a common practice in Montenegrin and Serbian nurseries; as such, the obtained results will contribute to the enhancement of nursery production of *Pinus nigra* and other conifers. This also could be assumed as a starting point for many further efforts and investigations with autochthonous fungal and plant material in this region.

Key words: nursery production; spore inoculums; vegetative inoculums; Submediterranean climate; *Pinus heldreichii*; *Pisolithus arhizus.*

Resumen

Micorrización de plantulas en contendor de *Pinus nigra* con *Suillus granulates* bajo condiciones de campo

La micorrización de plántulas actúa como una herramienta eficaz para la mejora de la calidad de los brinzales. En este estudio se investiga la eficacia de *Suillus granulatus,* procedentes de bosques de *Pinus heldreichii* (Montenegro), para producir plántulas ectomicorrícicas en contenedores de *Pinus nigra* autóctonos en condiciones de campo. Se ensayaron inoculacion por esporas (10^6, 10^7, 10^8) y vegetativa (1:16, 1:8, 1:4) para la formación ectomicorrízica y el crecimiento de las plántulas. Se utilizó inóculo de esporas y vegetativo de *Pisolithus arhizus* autóctonos en el mismo ensayo como tratamiento control.

La utilización de inóculos vegetativos y esporas de *S. granulatus* autóctonos ha demostrado ser un método eficaz para la obtención de plantas en contenedor ectomicorrícicas de *P. nigra* bajo condiciones de campo después de 11 meses. La inoculación de esporas de *S. granulatus* produjeron un buen desarrollo ectomicorrizas, disminuyendo el crecimiento de las plántulas de *P. nigra* en la primera estación de crecimiento. Las inoculaciones miceliales produjeron un escaso desarrollo ectomicorrizico de *S. granulatus,* con aumento en el crecimiento de las plántulas. Por lo tanto, sería factible el uso de inóculos de esporas de S. granulatus, con 10^6 esporas por planta, para producir plantas ectomicorrícicas de *P. nigra* a gran escala.

La inoculación micorrízica controlada de plántulas no es una práctica común en los viveros de Serbia y Montenegro y, como tal, los resultados obtenidos contribuirán a la mejora de la producción en viveros de *Pinus nigra* y otras coní-

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feras. Esto también podría ser asumido como un punto de partida para futuras investigaciones con material autóctono de hongos y plantas en esta región.

Palabras clave: producción de viveros; inóculo de esporas; inóculo vegetativo; clima submediterránea; *Pinus heldreichii; Pisolithus arhizus.*

Introduction

Afforestation programs in Montenegro and Serbia, proclaimed as one of the priorities in forest policy and for sustainable forest management (Anonymous, 2008), are nowadays generally targeted toward unfavourable, mostly carbonate, dry, infertile soils (Knežević *et al.*, 2011). Planting sites are located in different climate conditions, varying from Mediterranean and Submediterranean to dry continental climates, often on exposed hill slopes, characterized by extreme temperatures, low humidity, and low precipitation through the growing season. Containerized *Pinus nigra* Arn. seedlings are commonly used for afforestation (Stilinović, 1990), and general consensus is that the significance of pines in such programs here will be greater in the future. Therefore, the development of seedling production of pine species should be the primary goal of forestry science and commerce (Šijačić-Nikolić *et al.*, 2010).

Nursery practices are being focused on the production of seedlings for improving afforestation under the harsh environmental conditions. Mycorrhiza improves the mineral nutrition, growth, and adaptation of forest trees; therefore, seedling mycorrhization is thought to be among the most important tools for achieving higher seedling quality (Molina, 1979; Pera and Parlade, 2005; Ruiz-Diez *et al.*, 2006; Rincon *et al.*, 2007). In Montenegrin and Serbian nurseries, controlled mycorrhizal inoculation of seedlings has yet to become common practice, and the fungi and fungal isolates from this region have not been examined previously as the material for mycorrhization.

Suillus species are important fruiting ectomycorrhizal fungi in Mediterranean pine forests (Ruiz-Diez *et al.*, 2006), and *Suillus granulatus* (L.) Rousel sporocarps are among the most prominent fruiting ectomycorrhizal species in *Pinus heldreichii* H. Christ forests in Montenegro (Perić and Perić, 2006). Being tertiary relict and subendemic, *P. heldreichii* tends to occupy the extreme, xerothermic habitats of the mountain regions in the western part of the Balkan Peninsula, exposed to certain influences of the Mediterranean climate, where they form the upper forest line (Jovanović, 2007). Hypothesis that *S. granulatus*, originating from *P. heldreichii* forests, is adapted for dry, calcareous, exposed and poor habitats, being therefore suitable for *P. nigra* seedling mycorrhization was tested during this study.

To check the general set of environmental conditions for processing nursery ectomycorrhizal inoculations, *Pisolithus arhizus* (Scop.) Rauchert, originating from Montenegro, was tested as an additional control treatment. *P. arhizus* has been widely used in coniferous mycorrhization and is thought to be a promising inoculant (Marx *et al.*, 1982; Cairney and Chamber, 1997; Dunabeitia *et al.*, 2004; Pera and Parlade, 2005). It is rare and proposed to be protected in Montenegro (Perić and Perić, 2004), so its large-scale usage for pine seedling mycorrhization appears to be hardly achievable at this time.

The objectives of the present study were i) to enable identification of fungi and molecular characterization of isolates used for inoculation of seedlings; ii) to determine the capability of *S. granulatus* to inoculate *P. nigra* seedlings under open field conditions; iii) to compare the effects of spore and mycelial inoculation; and iv) to compare the efficiency of different concentrations of both types of inoculums on ectomycorrhizal formation and seedling growth.

Material and Method

Fungal material

Sporocarps of *Suillus granulatus* were collected in a *Pinus heldreichii* forest, above 1,500 m in altitude (42° 28’ 50.4’’ N; 019° 30’ 04.2’’ E’, loc. Kučka Korita). *Pisolithus arhizus* was collected beneath the more than 50-year-old *Cedrus atlantica* (Endl.) Manetti ex Carriere in Podgorica (42° 26’ 39.9’’ N; 19° 15’ 00.1’’ E, altitude 50 m). Identifications were carried out according to the keys of Munnoz (2005) and Sarasini (2005).

Isolation from the sporocarps was conducted on modified Melin-Norkrans (MMN) medium (Marx,

1969) amended with 50 ppm Streptomycin (Galenika, Serbia) and Ampicillin (Panfarma, Serbia) and 5 ppm Benomyl (Zorka, Serbia). The cultures were incubated at 22°C for 15-20 days in the dark. The isolates were transferred onto MMN medium and maintained by transfer every three months (Rincon *et al.*, 1999).

Molecular analysis

Fresh mycelium grown in liquid MMN medium of *S. granulatus* and *P. arhizus* was used for deoxyribonucleic acid (DNA) extraction, using a DNeasy Plant Mini Kit (Qiagen Ltd., USA). The internal transcribed region (ITS) of the ribosomal DNA was amplified using basidiomycete-specific primer pair ITS1-F and ITS4-B (Gardes and Bruns, 1993). The polymerase chain reaction (PCR) mixture contained 0.1 ng of DNA, 18.5 µL of sterile ultrapure water, 2 µL of each primer (10 pmol/µL), and 25 µL of Kapa Taq Ready Mix (Kapa, USA). Amplification conditions were based on Nieto and Carbone (2009): initial denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 0.5 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. A negative control was included.

The PCR products were purified with a QIAquick PCR Purification Kit (Qiagen Ltd., USA), and sequencing was performed by Macrogen Inc. (Seoul, Korea), utilizing ABI 3730 XL automated sequencer (Applied Biosystems, USA). Sequencing was performed in both directions, and raw sequence data were analyzed using BioEdit version 7.0.5.2 (Hall, 1999).

Comparison of sequences for ITS region was performed with BLAST-Blastn ver. 2.2.26 (Zhang *et al.*, 2000). Sequences were compared with the NCBI database (updated February 2012).

Alu I, *Hinf* I, *Mbo*I, and *Bsu*R (*Hinf* III) enzymes (Fermentas, EU) were used for RFLP analysis. The protocols of El Karkouri *et al.* (2002) were modified and used for RFLP analysis, as follows: 15 µl aliquots of the amplified ITS products were mixed with 1 µl (10 u) of enzyme, 2 µ of buffer, and 2 µl of deionized water, and incubated at 37°C for 12 hours. Restriction digest products were separated by electrophoresis on 2% agarose gels. Gels were stained with Midori green (Nippon Genetics, EU) and visualized using UV light. DNA ladder 100 bp (Nippon Genetics, EU) was used for fragment length estimation.

Production of fungal inocula

Vegetative inoculum was produced from mycelium grown in liquid MMN medium. After three months, the mycelium was transferred in 0.4-0.7 l glass yards with vermiculite saturated with MMN nutrient medium (glucose reduced to 2.5 g/l) and autoclaved (20 min, 120°C) (Rincon *et al.*, 1999).

Spore inoculum of *S. granulatus* was obtained from collected sporocarps (June 2009) and spore slurries were prepared from fragments of hymenium, which were triturated in sterile distilled water (Tores and Honrubia, 1994). The suspension was diluted in sterile water to reach the desirable spore concentration. Spore concentration was calculated with a haemacytometer.

For *P. arhizus* spore inoculum, dry basidiospores, obtained from sporocarps (September 2008) previously cleaned, dried, and stored under room temperature were mixed with vermiculite before application (Rincon *et al.*, 2001). Amount of spores which correlate with desirable spore concentration were used. One gram of *P. arhizus* spore dust contains 1.43×10^9 spores.

Plant material

Pinus nigra seeds were taken from the Užice Forest Seed Centre (loc. Šaranske šume UTM 34, X 381897, Y 4853549, Serbia). The seeds were soaked in water overnight and surface sterilized with 3% H_2O_2 for 30 min, and then rinsed with tap water, dried, and sowed (Landis, 1989).

Planting and maintenance

Seeds of *P. nigra* were sown in peat-vermiculite 1:1 (pH 5.8) under glass cover in QP 84T/11.5 trays (Quick Pot, HerkuPlast-Kubern GmbH, Germany). Two seeds were sown per cavity to ensure germination, thinned after five weeks to one per cavity. Seed sowing, germination, and initial plant development (from sowing to thinning, and one week more) were conducted under glass and, later, in open field conditions. The plants were shaded or partly shaded May-August, watered daily, and up to three times per week April-September. A fertilizer solution made up of 10 ml of 2 g/l Morton foliar fertilizer plus NPK 19: 9: 27 (ZIKO s.a., Greece) and 0.5 g/l Fertilion Combi 2 (COMPO GmbH & Co.KG, Germany) was applied by adding it to the plant

rhizosphere. There were six fertilizer treatments altogether (May 5-September 2), the first of which was without Fertilion Combi 2.

Experimental site conditions

The general climatic parameters for Podgorica could be summarized as follows: average year temperature of 15.5°C, with average seasonal temperatures of 14.3°C in spring, 25.1°C in summer (very hot), 16.0°C in autumn and 6.2°C in winter (mild). Average annual precipitation is 1,637.4 mm, but summer precipitation is only 10% of the total, with only 2% in July. Average yearly humidity is 64.7%, minimally 51.2% in July. Average annual duration of sunlight is 2,477.1 h, with 10.1 h daily during the summer months (69.1% of possible) (Burić *et al.*, 2007). Average temperature from the beginning of April to the end of August 2009 was 23.8°C, while total rainfall was 330.5 mm (data source: Hydrological and Meteorological Service of Montenegro).

Experimental design

Mycorrhizal synthesis was performed on *P. nigra* seedlings sown in March 2009. Twenty-eight plants per treatment were inoculated.

Different types of treatments were performed:

i) Seedlings treated with vegetative inoculum of *S. granulatus* at 1:16, 1:8, and 1:4 inoculum: substrate (v/v);

ii) Seedlings treated with spore inoculum of *S. granulatus* at 10^6, 10^7, and 10^8 spores per plant.

In order to check the general set of environmental conditions for the process of nursery ectomycorrhizal inoculations, *P. nigra* seedlings were treated with *P. arhizus*. Hence, different types of control treatments were set in the experiment:

i) Seedlings treated with vegetative inoculum of *P. arhizus* at 1:16, 1:8, and 1:4 inoculum: substrate (v/v);

ii) Seedlings treated with spore inoculum of *P. arhizus* at 10^6, 10^7, and 10^8 spores per plant;

iii) Water control-untreated seedlings of *P. nigra* growing under the same conditions.

The vegetative inocula of both fungi and *P. arhizus* spore inoculum were mixed with substrate for seed sowing. The containers were filled with inoculated substrata and sown with *P. nigra* seeds.

Inoculation with *S. granulatus* spore was applied on *P. nigra* seedlings in July in volumes of 10 ml per plant, two days after collecting the *S. granulatus* sporocarps.

Measured parameters

Preliminary controls of roots were conducted three months after seed sowing on the plants treated with vegetative inoculum of *S. granulatus,* and on the controlled seedlings treated with *P. arhizus*, on four randomly selected seedlings. Roots were observed under the stereomicroscope for fungal mantle and mycelium presence or absence, and were sectioned transversely for microscopic examination of Hartig net formation.

Eleven months after inoculation, the seedlings were harvested. The roots were washed free of substrate, and the plants were measured for stem height (mm) and root collar diameter (mm). The seedling shoots and roots were oven-dried (65°C, 48 h) to obtain the total dry weight, measured to 10^{-4} g accuracy. Root vs. shoot ratio was calculated. Twelve seedlings per treatment were analyzed.

Each seedling's root was cut into 2-3 cm segments, and the percentage of ectomycorrhizal short roots was assessed by counting at least 200 randomly selected short roots under the stereomicroscope (Parlade *et al.*, 1996; Rincon *et al.*, 2001). Grouping of the obtained results was made according to pre-set estimation ranges 1-3, as follows:

1. Root tips are simple and dichotomous. Multiple dichotomous tips rare, less than 20% of total. Mantle very thin and good extramatrical development is partly recorded.

2. Root tips are simple dichotomous and multiple dichotomous. Good extramatrical development of mycelia is evident.

3. Dichotomous and multiple dichotomous root tips prevail (more than 50% of total). Mantle layer is well established, with abundant extramatrical mycelium.

Microscopic examinations of Hartig net formation were principally performed on roots ranked 1, but also on those graded 2 and 3.

Statistical analysis

Data from the treatments were analyzed by one-way analysis of variance (ANOVA), and significant differences among treatments were separated by Tukey's B test ($p < 0.05$). Statistical analysis was performed with SPSS 10.0 for Windows (SPSS Inc., Chicago, USA).

Results

Molecular characterisation

According to the morphological features all collected fruit bodies were identified as either *Suillus granulates* or *Pisolithus arhizus*. Obtained ITS region sequences and RFLP patterns confirmed identification of isolates as *Suillus granulatus* and *Pisolithus arhizus* (Table 1), and could serve as efficient tool for their monitoring.

Seed germination, growth, and mycorrhization of seedlings

In the containers with substrata inoculation, germination started after 8-10 days, and in a 14-day period, about 80% of the seeds had germinated. In the containers without substrata inoculation, the seeds started germinating later, but within 21 days all the seeds had germinated. The percentage of seedling survival during the test was close to 97%.

Preliminary control of the seedling roots, conducted three months after seed sowing, on the plants treated with vegetative inoculum of *S. granulatus* and on the control seedlings treated with *P. arhizus*, showed a development of ectomycorrhiza on about 1/3 of the examined roots.

There were no statistically significant differences in growth of *P. nigra* seedlings for three spore and three vegetative inoculums treatments with *P. arhizus*, after 11 months (data not shown). Therefore, obtained data were analyzed as two groups: i) *P. arhizus*-vegetative and ii) *P. arhizus*-spore.

There were no statistically important differences among the treatments according to collar root diameter, root dry weight and root/shoot ratio of seedlings (Table 2). The seedlings treated with lower proportion (1:16, 1:8) of vegetative inoculum of *S. granulatus* have had longer shoots than the water control, while shoot length was significantly decreased by spore inoculation.

In comparison with *P. arhizus* inoculation, shoot height following the *S. granulatus* spore inoculation decreased. Vegetative inoculation with *S. granulatus* had pretty the same results as the *P. arhizus* spore inoculation, while the maximal shoot height is achieved with *P. arhizus* vegetative inoculation. Shoot dry weight in general follows the trend described for seedling height.

After 11 months, all the seedlings were colonized, and Hartig net was evident on nearly 100% of the mycorrhizal rootlets.

The degree of mycorrhizal development on seedling rootlets varied, according to visual estimation of ectomycorrhizal morphology (grades 1-3) (Table 2).

Vegetative inoculation formed poorly developed mycorrhiza. The root tips were single or dichotomous; multiple dichotomous root tips were rare. Mantle and extrametrical mycelium development around the roots and across the substratum is missing (grade 1, Table 2, Fig. 1A). After spore inoculation, multiple dichotomous root tips were abundant, as were development of mantle and extrametrical mycelium (grade 3, Table 2, Fig. 1B). Mycorrhizal tips are simple, dichotomous, and multiple dichotomous, generally straight, and short stiped. Extramatrical mycelia are loosely enveloped, cream to reddish-brownish in colour, and sometimes appear as a dense, cottony layer of mycelia through the root tips.

Table 1. Comparison of tested isolates sequences with NCBI database and fragment sizes observed after endonuclease restriction of ITS region

ITS sequence			BLAST (NCBI database)			RFLP fragment size (bp)			
GenBank acces.no	**Species**	**Lng (bp)**	**Closest accession**	**Id bp (%)**	**G bp (%)**	***Bsu*R**	***Hinf*I**	***Alu*I**	***Mbo*I**
JQ685727	*Suillus granulatus*	802	AY898617	794/799 (99)	2/799 (0)	460 240 120	205 120	685 120	270 255 130
JQ685724	*Pisolithus arhizus*	611	FM213365	608/611 (99)	1/611 (0)	750	280 265 190	560 180	290 190 155

Lng: length; Id: identity; G: gaps.

Table 2. *Pinus nigra* seedling growth characteristics and ectomycorrhiza development 11 months after inoculation with *Suillus granulatus*

Treatment		Collar root diameter	Shoot height	Shoot dry weight	Root dry weight	R/S	ECM	
		10^{-3} m		10^{-3} g			%	G
Suillus granulatus								
veg	1:16	1.85 ± 0.17^{a}	99.50 ± 13.3^{bc}	483.4 ± 42^{b}	268.0 ± 46^{a}	0.55 ± 0.08^{a}	100	1
	1:8	1.82 ± 0.10^{a}	99.83 ± 12.4^{bc}	426.5 ± 78^{ab}	254.9 ± 45^{a}	0.60 ± 0.06^{a}	100	1
	1:4	1.85 ± 0.20^{a}	93.83 ± 11.6^{b}	375.7 ± 37^{a}	226.8 ± 34^{a}	0.60 ± 0.07^{a}	100	1
spo	10^{6}	1.89 ± 0.22^{a}	75.58 ± 8.2^{a}	399.0 ± 92^{ab}	238.2 ± 50^{a}	0.61 ± 0.12^{a}	100	3
	10^{7}	1.86 ± 0.28^{a}	76.08 ± 9.1^{a}	372.7 ± 26^{a}	221.4 ± 50^{a}	0.60 ± 0.14^{a}	100	2
	10^{8}	1.93 ± 0.12^{a}	81.33 ± 11.6^{a}	386.1 ± 116^{ab}	230.3 ± 38^{a}	0.63 ± 0.15^{a}	100	3
Control								
P.a.	veg	1.91 ± 0.15^{a}	107.5 ± 15.1^{c}	484.7 ± 106^{b}	246.9 ± 52^{a}	0.52 ± 0.10^{a}	100	2
	spo	1.90 ± 0.17^{a}	99.58 ± 9.68^{bc}	461.6 ± 105^{ab}	257.2 ± 62^{a}	0.56 ± 0.10^{a}	100	3
water		1.78 ± 0.10^{a}	93.26 ± 7.42^{b}	403.3 ± 74^{ab}	247.0 ± 90^{a}	0.62 ± 0.09^{a}	66	*

Values (mean ± standard deviation); different letters in column indicate significant differences according to Tukey's B test ($p < 0.05$). R/S: root/shoot ratio; ECM ectomycorrhizal development on seedling roots; %: percentage of ectomycorrhizal short roots; G: estimation ranges for ectomycorrhiza development; veg: vegetative inoculum; spo: spore inoculum; P.a.: *Pisolithus arhizus;* * native ectomycorrhiza.

There was abundant development of mycorrhiza on the roots of the seedlings treated with *P. arhizus* spores (grade 3, Table 2, Fig 1D), and moderate development of mycorrhiza on the roots of the ones treated with mycelia (grade 2, Table 2, Fig 1C).

Poorly developed *S. granulatus* ectomycorrhiza had increased *P. nigra* seedlings growth, while in case of abundant ectomycorrhizal development, seedling growth was decreased. That is not the case with *P. arhizus*, where good developed ectomycorrhiza on roots did not affect, then encourage, seedlings growth.

The percentage of mycorrhizal rootlets in the water control seedlings was around 66%, and four types of ectomycorrhizas were detected. *Suillus* represents more than 25% of this mycorrhiza on one third of the analyzed seedlings, and the rest are unidentified native ectomycorrhizas. Native ectomycorrhizas were also present on some parts of the rootlets (10-30%) and on one quarter of the analyzed seedlings treated with vegetative inoculum of *S. granulatus*, but not on the seedlings treated with spores.

Discussion

Tests showed that vegetative and spore inoculum of *Suillus granulatus*, originated from *Pinus heldreichii* forests, was an effective method for obtaining containerized ectomycorrhizal *P. nigra* seedlings in open-field Submediterranean conditions.

It could be presumed that fungi from *P. heldreichii* forests are well adapted to the hard and extremely dry environmental conditions prevalent in their natural

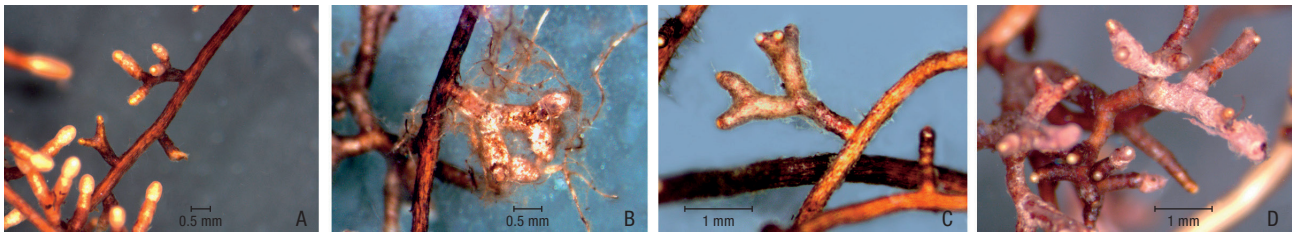

Figure 1. Ectomycorrhiza developed on *Pinus nigra* seedlings, 11 months after seed sowing and inoculation with *Suillus granulatus:* A. Grade 1-vegetative inoculum in 1:8 (v/v) proportion, B. Grade 3-spore inoculums, using 10^{6} spore/plan); *Pisolithus arhizus:* C. Grade 3-spore inoculum of using 10^{6} spore/plant, D. Grade 2-vegetative inoculum in 1:16 (v/v) proportion.

habitats, which was partly confirmed through this examination. In addition, the fungi produce abundant sporocarps, making it easy to obtain spore inoculums for large-scale application in forest nurseries (Marx *et al.,* 1982; Tores and Honrubia, 1994; Parlade *et al.*, 1996).

Controlled mycorrhizal inoculation programs in forest nurseries have been using the *Suillus* spp. for almost 30 years. Under the semi-arid Mediterranean environmental conditions, *S. collinitus* is thought to be a suitable and lasting inoculant for *Pinus halepensis* Mill. and *Pinus pinaster* Aiton (Tores and Honrubia, 1994; Pera and Parlade, 2005; Ruiz-Diez *et al.*, 2006; El Karkouri *et al.*, 2006; Rincon *et al.*, 2007). *S. granulatus* has been used for mycorrhization of *P. ponderosa* Douglas ex C. Lawson and *P. contorta* Douglas seedlings in North America (Riffle and Tinus, 1982; Clein and Reid, 1982) and for *P. halepensis* in Spain (Rincon *et al.*, 2005; Pera and Parlade, 2005). Investigations in culture (Sanchez *et al.*, 2001) showed that *S. collinitus* from Mediterranean forests grew at high water stress levels, and under high temperature conditions (30°C).

An investigation of fungal genomes, conducted by Manian *et al.* (2001), allowed treating *S. collinitus* and *S. granulatus* as closely related species. On the other hand, ectomycorrhizal fungi display extensive intraspecific variations in a range of physiological and other life-history parameters (Cairney, 1999), so the examination of each single isolate of fungi could be valuable, expressing their own specialties.

Restriction fragment analyses of ITS region were in this study used for species identification, but also for development of species specific markers, which will be used for the identification and monitoring of fungi, in programs of controlled seedling mycorrhization. Obtained RFLP patterns for *S. granulatus* and *P. arhizus*, following an isolation of fungal rDNA from mycelial culture, were similar to previously reported ones (Karen *et al.*, 1997; El Karkouri *et al.*, 2002; Gomes *et al.*, 1999; El Karkouri *et al.*, 2006; Wallender *et al.*, 2003), but not the same. According to Manian *et al.* (2001), the ITS regions among the *Suillus* spp. are clearly divergent, and *S. granulatus* shows a high level of intra-specific divergence for groups, based on geographical region.

The presented results show that mycelial inoculation with *S. granualtus*, followed with undeveloped fungal mantle, increasing seedling growth, while in case of spore inoculation, ectomycorrhizal mantle is good developed, but seedling growth is decreased. At the same environmental conditions, we also tested *P. arhizus* isolate from Montenegro. We used the results obtained herein as additional control treatment, with the idea of checking the overall set of environmental conditions for the process of ectomycorrhization. Inoculation of *P. nigra* seedlings with autochthonous *P. arhizus* (Lazarević, 2010) under the same planting conditions showed that mycelial inoculation, followed with a generally undeveloped ectomycorrhizal mantle, affected seedling growth in a similar manner as in the case of inoculation with *S. granulatus* mycelia. *P. arhizus* spore inoculation produces better developed mycorrhiza than inoculation with mycelia, without affecting the seedlings' growth. It is known that carbon metabolites, nutrients, and hormones are redirected from seedlings toward the symbiotic fungus, and that *Suillus* species have great demands for carbohydrates (Hacskaylo, 1973). If just the initial mycorrhization of roots with *S. granulatus* had been achieved, where the mycosymbiont had not been well established (using vegetative inoculum at 1:16 and 1:8), it would have influenced the increase of seedling growth. The two possible influences had been in equilibrium at one moment (1:4), but further development of the fungus, like the one following spore inoculation, decreased seedling growth.

Under the open field condition *S. granulatus* spore inoculation seems more efficient. All applied concentrations of spore inoculums were effective without differences. Minimal concentration necessary to ensure mycorrhization was not determined, due to fact that the lowest studied concentration of *S. granulatus* spore inoculum has shown good results.

It would be feasible to use spore inocula of *S. granulatus* with 10^6 spores per plant or less to produce ectomycorrhizal *P. nigra* plants. Moreover, spore inoculum could be recommended for large scale nursery production as easy for application, available in large quantities from sporocarps and not expensive (Parlade *et al.*, 1996; Rincon *et al.*, 2001).

Concentration necessary to ensure mycorrhization with vegetative inoculation of *S. granulatus* seems could not be lower than 1:16 under the open field conditions. Check after 22 months (data not presented) have shown that ectomycorrhiza in treatments with *S. granulatus* mycelia was well developed and abundant, with strong mycelium development throughout the substratum, suggesting the efficiency of applied inoculums concentrations. Two years after planting,

there were no statistically important differences among the treatments according to all measured parameters, except that the collar root diameter of non-treated seedlings was significantly smaller (data not shown).

According to the presented results optimal application rates of inoculums is influenced by environmental conditions during the growing season. Reddy and Natarajan (1997) reported that formation of ectomycorrhizas by fungus depends on temperature, pH, nutrients, moisture, aeration, external carbohydrates, and other abiotic factors, providing ample reasons for the variation in colonization of inoculated fungi. One should bear in mind that, during our tests conducted in the open field, environmental conditions were not optimal: temperatures in the substrates were often very high, and humidity conditions varied from wet to completely dry in one or two days. We suppose that, due to this reason, a complete ectomycorrhizal development for *S. granulatus* and *P.arhizus* was relatively slow and extended. More stable environmental conditions in the process of plant production will contribute to better development of the ectomycorrhizal mantle and of fungal growth (Castellano and Molina, 1989).

According to overall mantle development, it looks that harsh environmental condition affecting the development of *S. granulatus* less than *P. arhizus*, if applied as spore inoculum. After 11 months, in comparison with *P. arhizus*, mycorrhiza formed by *S. granulatus* were characterised with much more abundant rhizomorphs and mycelia strands, which are, according to Parlade *et al.* (1996) and Agerer (2001), structure of importance for water transport to the host plant.

Diverse experiments, carried out in field conditions around the world with different tree species, have provided varying results; in some cases, there was no growth stimulation of the seedlings (Castelano and Molina, 1989; Castellano 1994, 1996). It has been observed that there is no positive correlation between increase in growth of containerized pine seedlings exhibiting abundant ectomycorrhiza in the early stage of seedling development (Marx *et al.*, 1982; Clein and Reid, 1982) and the growth of pines, spruces, and firs that were frequently unaffected or depressed, instead of stimulated (Castellano and Molina, 1989).

Although *S. granulatus* spore inoculations decrease seedling growth, the treatments we applied could be considered effective. Studies related on enhancement of resistance to environmental extremes have confirmed that ectomycorrhizal fungi improve the survivorship of young trees (Marx *et al.*, 1982, Rincon *et al.*, 2007). It is frequently observed that more severe the environmental conditions were, the greater would be the requirement for mycorrhizal colonization in order to assure survival (Marx *et al.*, 1982). Well-developed ectomycorrhiza on seedlings roots would have efficiently absorbed water and nutrients from the greater volume of forest soil at the moment of plant establishment in the field, having a positive effect on seedling survival, growth, and reproduction (Parlade *et al.*, 1996; Berman and Bledsoe, 1998).

Further studies are needed to establish the minimal application rate for *S. granulatus* spore inoculations of *P. nigra* containerized seedlings in commercial nurseries and field plantations. Long-term field experiments are needed to compare the effects of different treatments, as well as treatments with different ectomycorrhizal fungi on the survival and growth of outplanted seedlings. All these studies are aimed toward selecting ectomycorrhizal fungi and inoculation methods, as well as to complete culture practices suitable for improving standard reforestation practices with *P. nigra* in Montenegro and regions of Southeastern Europe.

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